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13. ABSTRACT ***(Maximum 200 Words)***

The p53 tumor suppressor gene mediates a pathway leading to G1 cell cycle arrest and/or apoptosis. p53 function is frequently altered in breast cancer and other human cancers. p53 protein is kept at a low level during normal cell growth by its short half-life and is stabilized following oncogenic stimulation and DNA damage. This research is exploring factors that contribute to the stabilization of p53 after oncogenic stimulation. MDM2 oncoprotein binds to and targets p53 for degradation in the cytoplasm. The tumor suppressor ARF stabilizes p53 by blocking the nuclear export of both p53 and MDM2. In work under this award, it was shown that ARF has biological activity independent of p53. Overexpression of ARF impedes DNA synthesis, resulting in accumulation of cells in S phase. Hence, ARF induction induces predominantly G1 arrest if p53 is present or S-phase retardation in the absence of p53. The Principal Investigator (PI) also obtained evidence that p53 protein contains two nuclear exporting signal sequences, one specific for MDM2 and one whose activity is independent of MDM2.

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RE: ANNUAL REPORT FOR DAMD 17-99-1-9574

PI: YUE XIONG, PH.D.

INTRODUCTION

The p53 tumor suppressor gene mediates a major tumor suppression pathway in mammalian cells that is frequently altered in human cancers (Levine, 1997; Prives, 1998; Sherr, 1998). p53 protein is kept at low level during normal cell growth by its short half-life and is stabilized following oncogenic stimulation and DNA damage. Growing evidence has identified MDM2 as a key regulator of p53 protein stability (Haupt et al., 1997; Kubbutat et al., 1997). Blocking p53 nuclear exporting leads to p53 stabilization (Roth et al., 1998; Freeman et al., 1998), indicating that p53 degradation occurs in the cytoplasm and suggesting p53 nuclear export as a major regulatory event in MDM2-mediated p53 degradation. The tumor suppressor ARF stabilizes p53 by blocking the nuclear export of both p53 and MDM2 (Tao and Levine, 1999; Zhang and Xiong, 1999). Human tumor-derived mutations in the *ARF-INK4a* locus can selectively target the *ARF* gene and impair ARF function in blocking p53 nuclear export, underscoring the importance of nuclear export in regulating p53 stability (Zhang and Xiong, 1999). The mechanism governing p53 nuclear export has been under intense investigation, but remains unclear and controversial at present.

The mammalian ARF-INK4a locus uniquely encodes two cell cycle inhibitors, $p16^{INK4a}$ (Serrano et al., 1993) and ARF (Quelle et al., 1995), by using separate promoters and alternative reading frames, that both function in tumor suppression. Mice with homozygous deletion of both p16 and ARF (Serrano et al., 1996), or selective deletion of ARF, sparing p16 (Kamijo et al., 1997), develop spontaneous tumors at an early age. $p16^{INK4a}$ encodes a specific inhibitor of CDK4/CDK6 and maintains the retinoblastoma protein in its growth suppressive state through inhibition of its phosphorylation(Serrano et al., 1993), while ARF binds to and inactivates MDM2, thereby stabilizing p53 (Pomerantz et al., 1998; Zhang et al., 1998; Stott et al., 1998). MDM2 binds to and abrogates p53 function by either targeting p53 for degradation in the cytoplasm or by repressing p53's transcriptional activity in the nucleus [reviewed in (Levine, 1997; Prives, 1998)]. ARF inhibits MDM2 at least in part by preventing MDM2-dependent nuclear export and cytoplasmic degradation of p53 (Zhang and Xiong, 1999; Tao and Levine, 1999), through forming ARF-MDM2-p53 ternary complex (Zhang and Xiong, 1999).

We showed previously that ARF forms nuclear bodies with MDM2 and p53 and blocks p53 and MDM2 nuclear export, thereby inhibiting MDM2-mediated p53 degradation (Zhang and Xiong, 1998). Like oncogenic signals, DNA damage also activate p53, but through an ARF-independent pathway that involved p53 protein phosphorylation by several DNA damage-activated kinases. One of the major findings we made over the past year is that DNA damage pathway also causes p53 stabilization through regulating p53 nuclear export.

KEY RESEARCH ACCOMPLISHMENT

A p53 Amino Terminal Nuclear Export Signal Inhibited by DNA Damage-Induced Phosphorylation

The p53 gene mediates a major tumor suppression pathway that is frequently altered in human cancers (Levine, 1997). p53 is inhibited during normal cell growth by MDM2, a proto-oncogene discovered by its genomic amplification on a murine double minute chromosome, through either ubiquitin-dependent p53 degradation in the cytoplasm, or repression of p53's transcriptional activity in the nucleus. p53 is activated following DNA damage through p53 phosphorylation (Prives, 1998), or in response to oncogenic insults by the activation of ARF, a tumor suppressor encoded by the alternative reading frame of the INK4a locus that is frequently altered in human cancers (Sherr, 1998). Blocking p53 nuclear export leads to p53 stabilization and activation (Roth et al., 1998; Freedman et al., 1998), and ARF stabilizes p53, in part, by blocking the nuclear export of both p53 and MDM2 (Zhang and Xiong, 1998), underscoring the importance of nuclear export in regulating p53 stability.

The N-terminal sequence in p53 is required for its nuclear export

To clarify the role of MDM2 in p53 nuclear export and discriminate between these models, we first examined the sensitivity to MDM2-mediated degradation and nuclear export of p53 harboring either L14Q/F19S or L22Q/W23S mutations in its N-terminal region. To our surprise, despite containing the intact NES in the COOH-terminal region (Stott et al. 1998), both $p53^{L14Q/F19S}$ and $p53^{L22Q/W23S}$ exhibited significantly decreased nuclear export activity, from 73% seen with wild type p53 to 23% and 15% with the two mutants. Thus, the COOH-terminal located NES was required, but not sufficient, for p53 nuclear export. Supporting the idea that the inhibitory effect of L22Q/W23S mutation on p53 export may be reduced or even masked by the overproduction of p53, endogenous $p53^{L22Q/W23S}$ protein produced by a knock-in strategy that was expressed at a lower level is more completely blocked from nuclear export and accumulates to very high levels in the nucleus, providing in vivo evidence for the disruption of p53 nuclear export by L22Q/W23S mutations.

MDM2 is not required for p53 nuclear export

Blockage of p53 nuclear export by the L14Q/F19S and L22Q/W23S mutations could be attributed to the disruption of p53-MDM2 association if MDM2 shuttled p53 out of the nucleus (Roth et al. 1998) or if MDM2 was required for p53 nuclear ubiquitination and its subsequent export (Boyd et al. 2000; Geyer et al. 2000). Alternatively, it could be attributed to an intrinsic property of the NH_2-terminal domain of p53. We carried out a series of heterokaryon assays to determine if p53 nuclear export was dependent on MDM2. After examining a large number of heterokaryons, we did not observe apparent differences in p53 nuclear export efficiency in the absence of HDM2 or in the presence of high levels of HDM2. To exclude the possibility that trace amounts of HDM2 in the Saos-2 cells might have traveled to $p53^{-/-}$-$MDM2^{-/-}$ MEFs and promoted p53 export, we performed a similar heterokaryon assay in which $p53^{-/-}$-$MDM2^{-/-}$ MEFs were first infected with p53-expressing adenoviruses and then fused with Saos-2 cells that had been infected with ARF-expressing adenoviruses to block MDM2 export. In these experiments, p53 shuttled from $p53^{-/-}$-$MDM2^{-/-}$ MEFs to ARF-overexpressing Saos-2 cells in majority of the heterokaryons examined. In a separate experiment, p53 was expressed in one set of $p53^{-/-}$-$MDM2^{-/-}$

MEFs, and then fused with another set of $p53^{-/-}$-$MDM2^{-/-}$ MEFs that had been infected with Ad-hARF and identified by an antibody specifically recognizing human, but not mouse, ARF. In a majority (84%) of the heterokaryons formed by fusion of ARF-negative (Ad-p53 infected) and ARF-positive (uninfected) $p53^{-/-}$-$MDM2^{-/-}$ MEFs, active p53 nuclear export was clearly observed. The efficiency of p53 nuclear-cytoplasmic shuttling between two murine nuclei through the murine cytoplasm is comparable to that seen between human and mouse nuclei through a mixed human/mouse cytoplasm. Thus, p53 nuclear export was not dependent on the presence of MDM2, and impaired p53 nuclear export by the L14Q/F19S and L22Q/W23S mutations is likely to be caused by a mechanism other than the disruption of p53-MDM2 association.

The p53 N-terminus contains a functional NES

The NH_2-terminal region of p53 contains two hydrophobic, leucine-rich stretches which resemble the CRM1-dependent NESes found in several different proteins including MDM2 and p53. The first leucine-rich stretch is highly conserved between human and mouse p53 (15 out 17 residues identical) while only 8 out of 18 residues in the second leucine-rich sequence are conserved. Notably, the L14Q/F19S and L22Q/W23S mutations each alter a highly conserved hydrophobic Leu residue shown to be critically important for NES function in cAMP-dependent protein kinase inhibitor, PKI, HDM2, and p53. We attached both $p53^{11-27}$ and $p53^{31-47}$ peptides to GFP and determined whether these leucine-rich motifs function as an autonomous NES. GFP distributes throughout both nucleus and cytosol, with slightly more accumulation in the nucleus. Fusion of GFP with the NES from either HDM2 or the p53 C-terminus ($p53^{cNES}$) resulted in evident nuclear exclusion of fluorescence in about two-thirds of the cells, confirming the nuclear export activity of both NESes. Attachment of the peptide from p53 residues 11–27 ($p53^{nNES}$) to GFP generated clear nuclear exclusion of GFP in about two-thirds of the cells, a comparable efficiency with the NESes from HDM2 and the p53 C-terminus. The nuclear exclusion of GFP by fusion with all three NESes is, however, incomplete and nuclear fluorescence remains visible. This is likely due to the combination of weak NES activity, and visualization of cells as a plane, as opposed to three dimensionally, with the consequent inability to exclude cytoplasmic fluorescence surrounding the nucleus as non-nuclear signals. Fusion with the $p53^{31-47}$ peptide only weakly affected GFP distribution and less than one-quarter of the GFP-positive cells exhibited a slight cytoplasmic accumulation of GFP. The significance of this weak nuclear export activity, if genuine, is difficult to assess and remains unclear at present. Inhibition of CRM1 by LMB treatment reverted the nuclear exclusion of GFP fused with $HDM2^{NES}$, $p53^{cNES}$, and $p53^{nNES}$ to that of GFP alone, indicating that nuclear export by these three NESes was CRM1-dependent. L14Q/F19S and L22Q/W23S mutations prevented nuclear exclusion of $p53^{nNES}$-GFP, reinforcing the functional importance of these hydrophobic residues and the authenticity of the p53 nNES. The nuclear export activity of the $p53^{nNES}$, as well as that of the $p53^{cNES}$ and the PKI^{NES}, was not detectably affected by the high level of ARF expression. Thus, the NH_2-terminal domain of p53 appears to contain an autonomous NES that functions through the CRM1-dependent nuclear export pathway and is not dependent on MDM2.

DNA damage-induced phosphorylation at Ser-15 inhibits p53 nuclear export

The NH_2-terminal p53 NES contains two serine residues, Ser^{15} and Ser^{20}, adjacent to Leu^{14} and Leu^{23}. Phosphorylation of both Ser^{15} and Ser^{20} by several DNA-damage-activated p53 kinases has been linked to p53 stabilization and activation. To directly test the idea that phosphorylation at Ser^{15} and Ser^{20} in p53 might impair the activity of the N-terminal NES, we determined nucleo-cytoplasmic shuttling of

phosphorylated p53 induced by DNA damage and fund that ectopically expressed Ser^{15}-phosphorylated p53 was inhibited from exporting. To determine whether endogenously expressed, Ser^{15}-phosphorylated p53 is similarly blocked from export, U-2OS cells were untreated or irradiated with UV and then fused with Saos-2 cells. Almost no p53 was detected in untreated U-2OS cells. Upon UV irradiation, there was a substantial increase in total and Ser^{15}-phosphorylated p53. Ser^{15}-phosphorylated p53 was localized mostly to speckles and exhibited a pattern distinct from that detected by the anti-full length p53 antibody, suggesting that phosphorylation of p53 is associated with or promotes a change of p53 subnuclear localization. While there was active p53 nuclear export as determined by the use of anti-full length p53 antibody, no Ser^{15}-p53 signal was detected in the recipient Saos-2 cells (more than 30 heterokaryons examined). UV irradiation similarly resulted in a substantial increase in total and Ser^{15}-phosphorylated p53 in MEFs. Consistently, Ser^{15}-phosphorylated p53 was completely blocked from nuclear export.

REPORTABLE OUTCOMES

Supported in part by the DAMD17-99-1-9574, we have discovered a novel regulation of tumor suppressor p53 that p53 contains a novel NES that is inhibited by DNA damage-induced phosphorylation. These results have resulted in a publication of a research paper in journal *Science* (Zhang and Xiong, 2001a) and a review article in journal of *Cell Growth & Differentiation* (Zhang and Xiong, 2001a). A reprint of both papers is enclosed.

CONCLUSIONS

Our results reveal a previously unrecognized NES in the NH_2-terminal region of p53 that is required for the nuclear export of p53. The two separate NESes of p53 can functionally collaborate with each other and synergistically mediate protein nuclear export. Having two separate NESes could provide cells with greater versatility in regulating p53 export. The COOH-terminal p53 NES is situated within the tetramerization domain, leading to the postulation that regulated p53 tetramerization occludes this NES, thereby ensuring nuclear retention of the active DNA-binding form of p53. The NH_2-terminus of p53 contains several sites whose phosphorylation by various DNA-damage-activated p53 kinases lead to p53 stabilization and activation. Phosphorylation at Ser^{15} and Ser^{20} has been suggested to cause p53 stabilization by hindering p53-MDM2 binding. As p53 can undergo active nuclear export in the absence of MDM2, inhibition of MDM2 binding, while preventing p53 degradation, would not block p53 nuclear export and thus would not efficiently accumulate p53 in the nucleus to allow maximal p53 activation. On the other hand, inhibiting p53 nuclear export without breaking its binding with MDM2, although causing the nuclear accumulation of p53, would not reach maximal p53 activation either because MDM2, in addition to its activity in promoting cytoplasmic p53 degradation, can also directly inhibit p53's transactivating activity in the nucleus. We suggest that DNA damage-induced phosphorylation may achieve optimal p53 activation through the additive and complementary action of both inhibiting MDM2 binding to, and nuclear export of, p53.

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APPENDICES

None

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Science

A p53 Amino-Terminal Nuclear Export Signal Inhibited by DNA Damage–Induced Phosphorylation

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A p53 Amino-Terminal Nuclear Export Signal Inhibited by DNA Damage–Induced Phosphorylation

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The p53 protein is present in low amounts in normally growing cells and is activated in response to physiological insults. MDM2 regulates p53 either through inhibiting p53's transactivating function in the nucleus or by targeting p53 degradation in the cytoplasm. We identified a previously unknown nuclear export signal (NES) in the amino terminus of p53, spanning residues 11 to 27 and containing two serine residues phosphorylated after DNA damage, which was required for p53 nuclear export in colloboration with the carboxyl-terminal NES. Serine-15–phosphorylated p53 induced by ultraviolet irradiation was not exported. Thus, DNA damage–induced phosphorylation may achieve optimal p53 activation by inhibiting both MDM2 binding to, and the nuclear export of, p53.

The gene encoding p53 mediates a major tumor suppression pathway that is frequently altered in human cancers (*1*). p53 is inhibited during normal cell growth by MDM2, a proto-oncogene discovered by its genomic amplification on a murine double minute chromosome, through either ubiquitin-dependent p53 degradation in the cytoplasm (*2*) or repression of p53's transcriptional activity in the nucleus (*3*, *4*). p53 is activated after DNA damage through p53 phosphorylation (*5*, *6*), or in response to oncogenic insults by the activation of ARF, a tumor suppressor encoded by the alternative reading frame of the INK4a locus that is frequently altered in human cancers (*7*). Blocking p53 nuclear export

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leads to p53 stabilization and activation (*8*, *9*), and ARF stabilizes p53, in part, by blocking the nuclear export of both p53 and MDM2 (*10*, *11*), underscoring the importance of nuclear export in regulating p53 stability.

The mechanism governing p53 nuclear export remains unclear, and three competing models have been proposed (*12*). MDM2 may bind p53 in the nucleus and shuttle it to the cytoplasm (*8*, *13*), p53 may use a COOH-terminal–located nuclear export signal (NES) to mediate its own nuclear export (*14*), or MDM2 could ubiquitinate p53 in the nucleus to promote its nuclear export (*15*, *16*). To clarify the role of MDM2 in p53 nuclear export and discriminate between these models, we first examined the sensitivity to MDM2-mediated degradation and nuclear export of p53 harboring either L14Q/F19S ($Leu^{14} \rightarrow Gln$; $Phe^{19} \rightarrow Ser$) or L22Q/W23S ($Leu^{22} \rightarrow Gln$; $Trp^{23} \rightarrow Ser$) mutations (*17*). Consistent with previous reports (*2*), both $p53^{L14Q/F19S}$ and $p53^{L22Q/W23S}$ were resistant to degradation by HDM2 (human homolog of MDM2) (Fig. 1A). The inhibition of HDM2-mediated p53 degradation by these mutations could not be attributed to the loss of p53 transcriptional activity, because a mutation in the sequence-specific DNA binding domain, R273H ($Arg^{273} \rightarrow His$), which also abolishes p53 transcriptional activity, had no detectable effect on p53 stability (*18*). Despite containing the intact NES in the COOH-terminal region (*14*), both $p53^{L14Q/F19S}$ and $p53^{L22Q/W23S}$ exhibited substantially decreased nuclear export activity, from 73% with wild-type (WT) p53 to 23% and 15%, with the two mutants, respectively (Fig. 1, B and C). The transcriptionally inactive $p53^{R273H}$ was exported at an efficiency indistinguishable from that of WT p53 (Fig. 1B). Thus, the COOH-terminal–located NES was required, but was not sufficient, for p53 nuclear export. This result is different from a previous report of normal nuclear export of a $p53^{L22Q/W23S}$–green fluorescent protein (GFP) fusion (*14*). Several experimental differences in that study could have contributed to the discrepancy. These include the following, a four times longer cell fusion period, which would result in prolonged protein export; the electroporation of 10 times more plasmid DNA, which would result in a higher level of p53 protein; and the attachment of a GFP moiety that might have increased cytoplasmic retention of $p53^{L22Q/W23S}$-GFP, which would have then entered the mouse nuclei in the heterokaryons and scored as positive nuclear export. Supporting the idea that the inhibitory effect of the L22Q/W23S mutation on p53 export may be reduced or even masked by the overproduction of p53, endogenous $p53^{L22Q/W23S}$ protein produced by a knock-in strategy that was expressed at a lower level is more completely blocked from nuclear export and accumulates to very high levels in the nucleus [(*19*), Web fig. 1 (*20*)], providing in vivo evidence for the disruption of p53 nuclear export by L22Q/W23S mutations.

Blockage of p53 nuclear export by the L14Q/F19S and the L22Q/W23S mutations could be attributed to the disruption of p53-MDM2 association if MDM2 shuttled p53 out of the nucleus (*10*) or if MDM2 was required for p53 nuclear ubiquitination and its subsequent export (*15*, *16*). Alternatively, it could be attributed to an intrinsic property of the NH_2-terminal domain of p53. We carried out a series of heterokaryon assays to determine if p53 nuclear export was dependent on MDM2. After examining a large number of heterokaryons, we did not observe apparent differences in p53 nuclear export efficiency in the absence of HDM2 or in the presence of high levels of HDM2 [Web fig. 2 (*20*)]. To exclude the possibility that

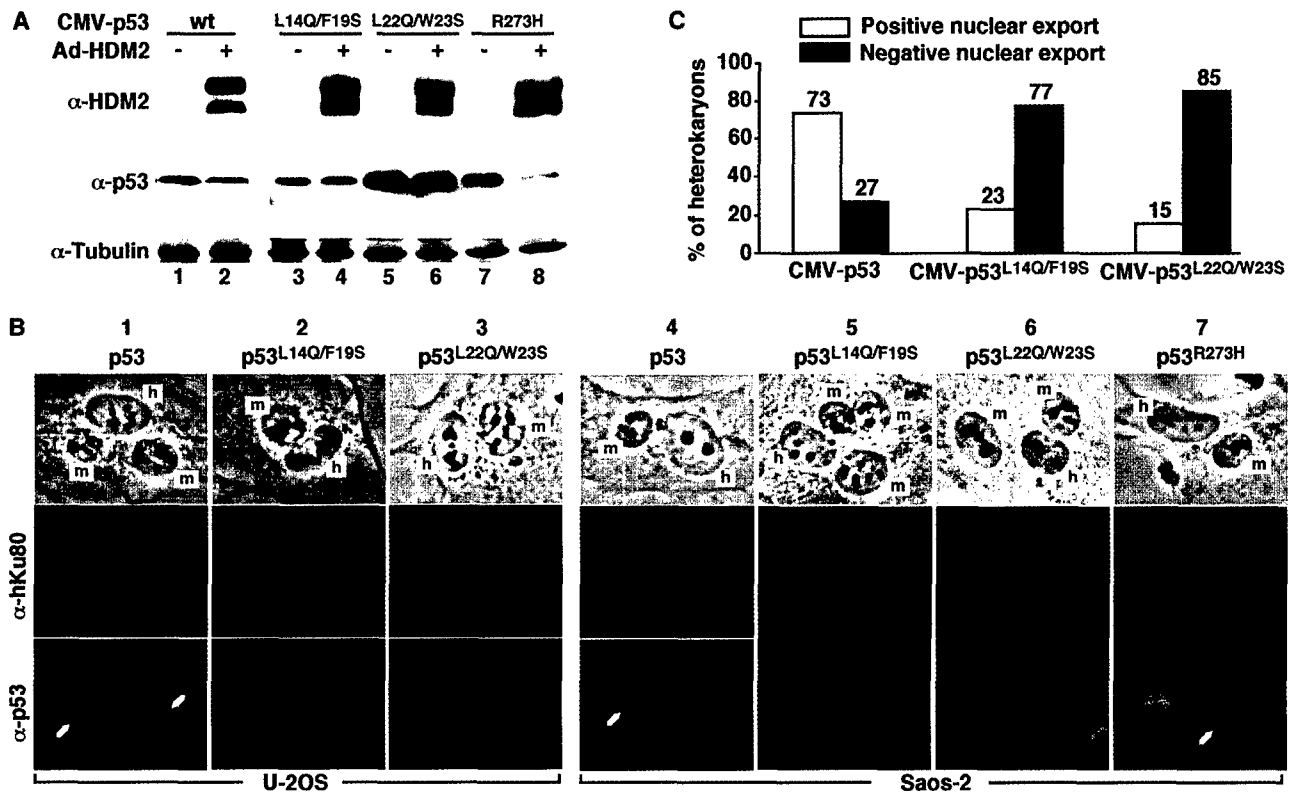

Fig. 1. p53 nuclear export is impaired by mutations in the NH_2-terminal domain. (**A**) Saos-2 cells were infected with Ad-HDM2 and transfected with individual plasmids expressing the indicated p53 protein. Whole-cell lysates from each infected-transfected cell population were separated by SDS–polyacrylamide gel electrophoresis and immunoblotted with antibodies to HDM2 and p53. (**B**) Human Saos-2 and U-2OS cells were transfected with plasmid DNA expressing the indicated p53 proteins. Twenty hours after transfection, cells were fused with $MDM2^{-/-}$-$p53^{-/-}$ MEF cells while de novo protein synthesis was inhibited. Forty-five minutes after fusion, cells were fixed and stained with the indicated antibodies. Arrows indicate positive p53 nuclear export. Nuclei of human (h) or mouse (m) origin are indicated in the phase-contrast images. (**C**) Fifty heterokaryons were counted from each transfection. The appearance of p53 in the mouse (Ku-negative) nuclei was scored as positive p53 nuclear export.

trace amounts of HDM2 in the Saos-2 cells might have traveled to $p53^{-/-}$-$MDM2^{-/-}$ mouse embryo fibroblasts (MEFs) and promoted p53 export, we performed a similar heterokaryon assay in which $p53^{-/-}$-$MDM2^{-/-}$ MEFs were first infected with p53-expressing adenoviruses and then fused with Saos-2 cells that had been infected with ARF-expressing adenoviruses to block MDM2 export (*10*, *11*). In these experiments, p53 shuttled from $p53^{-/-}$-$MDM2^{-/-}$ MEFs to ARF-overexpressing Saos-2 cells in a majority (76%) of the heterokaryons examined (Fig. 2, A and C). In a separate experiment, p53 was expressed in one set of $p53^{-/-}$-$MDM2^{-/-}$ MEFs, and then fused with another set of $p53^{-/-}$-$MDM2^{-/-}$ MEFs that had been infected with adenovirus expressing hARF (Ad-hARF) and identified by an antibody specific to human, but not mouse, ARF. In a majority (84%) of the heterokaryons formed by fusion of ARF-negative (Ad-p53 infected) and ARF-positive (uninfected) $p53^{-/-}$-$MDM2^{-/-}$ MEFs, active p53 nuclear export was observed (Fig. 2, B and C). The efficiency of p53 nuclear-cytoplasmic shuttling between two murine nuclei through the murine cytoplasm is comparable to that seen between human and mouse nuclei through a mixed human/mouse cytoplasm. Thus, p53 nuclear export was not dependent on the presence of MDM2, and impaired p53 nuclear export by the L14Q/F19S and L22Q/W23S mutations is probably caused by a mechanism other than the disruption of p53-MDM2 association. The ability of p53 to export independently of MDM2 does not exclude the possibility that p53 nuclear export may be affected by the function of MDM2.

The NH_2-terminal region of p53 contains two hydrophobic, leucine-rich stretches that resemble the chromosomal region maintenance 1 (CRM1)–dependent NESs found in several different proteins including MDM2 and p53 (Fig. 3A). The first leucine-rich stretch is highly conserved between human and mouse p53 (15 out of 17 residues are identical) whereas only 8 out of 18 residues in the second leucine-rich sequence are conserved. Notably, the L14Q/F19S and L22Q/W23S mutations each alter a highly conserved hydrophobic Leu residue shown to be critically important for NES function in cyclic adenosine 3′, 5′-monophosphate (cAMP)–dependent protein kinase inhibitor (PKI) (*21*), HDM2 (*8*), and p53 (*14*). We attached both $p53^{11-27}$ and $p53^{31-47}$ peptides to GFP and determined whether these leucine-rich motifs function as an autonomous NES. GFP distributes throughout both the nucleus and the cytosol, with slightly more accumulation in the nucleus (Fig. 3, B and C). Fusion of GFP with the NES from either HDM2 or the p53 COOH-terminus ($p53^{cNES}$) resulted in evident nuclear exclusion of fluorescence in about two-thirds of the cells (Fig. 3, B and C), confirming the nuclear export activity of both NESs. Attachment of the peptide from p53 residues 11 to 27 ($p53^{nNES}$) to GFP generated clear nuclear exclusion of GFP in about two-thirds of the cells, an efficiency comparable with that of NESs from HDM2 and the p53 COOH-terminus. The nuclear exclusion of GFP by fusion with all three NESs is, however, incomplete, and nuclear fluorescence remains visible. This is probably due to the combination of weak NES activity [see Web fig. 3 for quantitative comparison (*20*)] and visualization of cells as a plane, as opposed to three-dimensionally, with the consequent inability to exclude cytoplasmic fluorescence surrounding the nucleus as nonnuclear signals. Fusion with the $p53^{31-47}$ peptide only weakly affected GFP distribution, and less than one-quarter of the GFP-positive cells exhibited a slight cytoplasmic accumulation of GFP. The significance of this weak nuclear export activity, if genuine, is difficult to assess and remains unclear at present. Inhibition of CRM1 by leptomycin B treatment reverted the nuclear exclusion of GFP fused with $HDM2^{NES}$, $p53^{cNES}$, and $p53^{nNES}$ to that of GFP alone (Fig. 3, B and C), indicating that nuclear export by these three NESs was CRM1-dependent. L14Q/F19S and L22Q/W23S mutations prevented nuclear exclusion of $p53^{nNES}$-GFP, reinforcing the functional importance of these hydrophobic residues and the authenticity of the

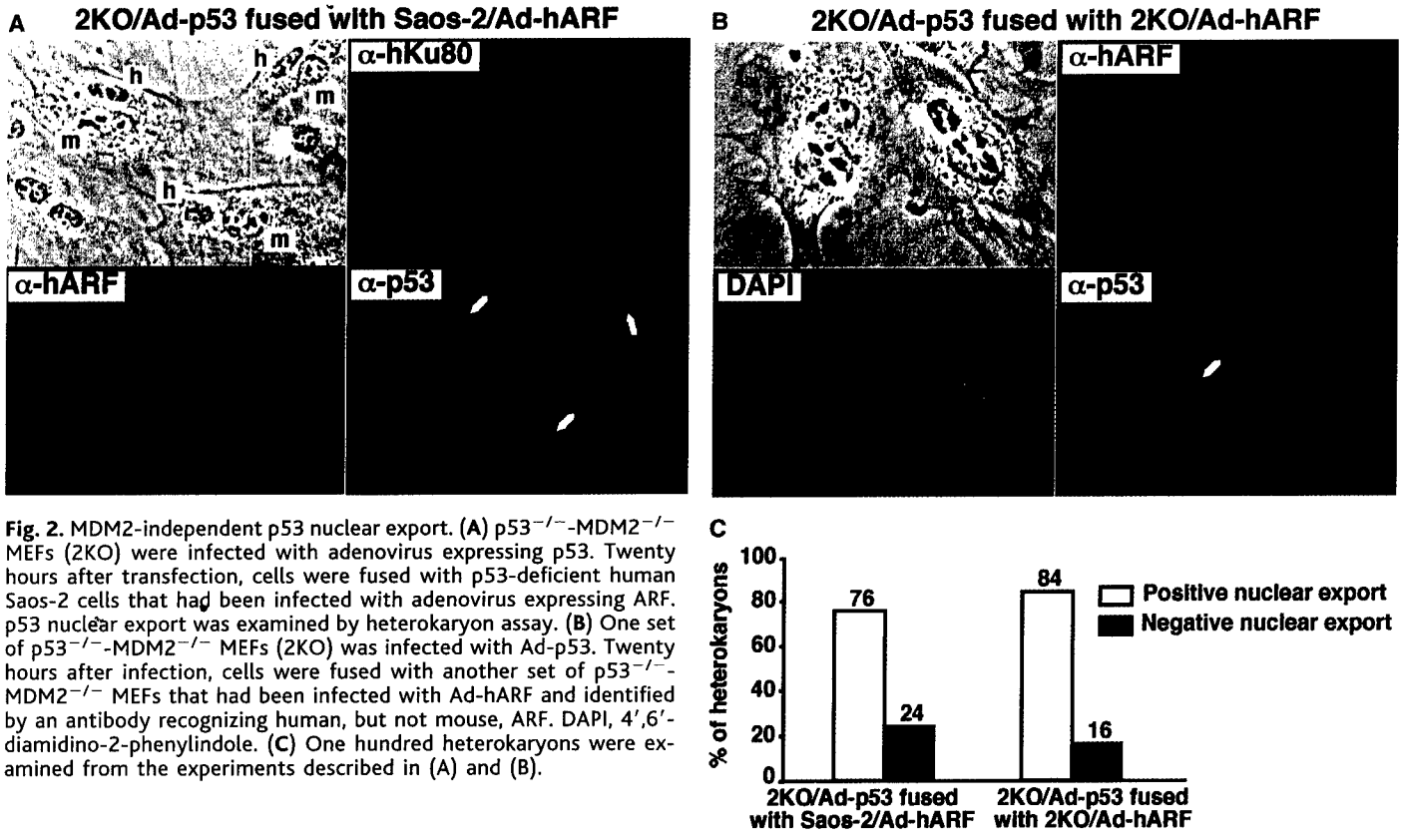

Fig. 2. MDM2-independent p53 nuclear export. (**A**) $p53^{-/-}$-$MDM2^{-/-}$ MEFs (2KO) were infected with adenovirus expressing p53. Twenty hours after transfection, cells were fused with p53-deficient human Saos-2 cells that had been infected with adenovirus expressing ARF. p53 nuclear export was examined by heterokaryon assay. (**B**) One set of $p53^{-/-}$-$MDM2^{-/-}$ MEFs (2KO) was infected with Ad-p53. Twenty hours after infection, cells were fused with another set of $p53^{-/-}$-$MDM2^{-/-}$ MEFs that had been infected with Ad-hARF and identified by an antibody recognizing human, but not mouse, ARF. DAPI, 4′,6′-diamidino-2-phenylindole. (**C**) One hundred heterokaryons were examined from the experiments described in (A) and (B).

p53 nNES. The nuclear export activity of the $p53^{nNES}$, as well as that of the $p53^{cNES}$ and the PKI^{NES}, was not detectably affected by the high level of ARF expression (Fig. 3D). Thus, the NH_2-terminal domain of p53 appears to contain an autonomous NES that functions through the CRM1-dependent nuclear export pathway and is not dependent on MDM2.

The NH_2-terminal p53 NES contains two serine residues, Ser^{15} and Ser^{20}, adjacent to Leu^{14} and Leu^{23}. Phosphorylation of both Ser^{15} and Ser^{20} by several DNA damage–activated p53 kinases has been linked to p53 stabilization and activation (*22*). A knock-in mutation at Ser^{18} of murine p53, which corresponds to Ser^{15} in human p53, delayed and reduced p53 accumulation and nearly completely abolished p21 accumulation in response to DNA damage (*23*). Substitution of both Ser^{15} and Ser^{20} with alanine residues did not cause any appreciable change in the nuclear and cytoplasmic distribution of fused GFP. However, the substitution of both Ser^{15} and Ser^{20} with aspartic acid residues, which often mimic the charge of a phosphorylated serine, decreased nuclear export activity, as judged by the lack of visible nuclear exclusion of fused GFP and an increase in the ratio of nuclear to cytoplasmic fluorescence (Fig. 4A). To directly test the idea that phosphorylation at Ser^{15} and Ser^{20} in p53 might impair the activity of the NH_2-terminal NES, we determined nucleo-cytoplasmic shuttling of phosphorylated p53 induced by DNA damage. Ad-p53–infected U-2OS cells were either left untreated or exposed to ultraviolet (UV) irradiation and then fused with Ad-ARF–infected Saos-2 cells. U-2OS/Saos-2 heterokaryons were identified by immunostaining with a mouse antibody to ARF. Nucleo-cytoplasmic shuttling of total or Ser^{15}-phosphorylated p53 was determined by using either a goat antibody against full-length human p53 (anti–full-length p53) or a rabbit antibody raised against a synthetic phospho-Ser^{15} p53 peptide (Fig. 4B). Anti–full-length p53 antibody detected high levels of p53 in almost all U-2OS cells (ARF negative) and lower amounts in the Saos-2 cells (ARF positive) present in the heterokaryons, but not in unfused Saos-2 cells, confirming the expression of p53 in U-2OS cells and its shuttling to the recipient Saos-2 cells. Anti–phospho-Ser^{15} p53 detected a considerable amount of p53 in UV-irradiated, but not in untreated, cells or UV-treated $p53^{-/-}$ MEFs, confirming the specificity of the antibody in recognizing only the phosphorylated form of p53. Ser^{15}-phosphorylated p53 was not detected in the recipient Saos-2 cells fused with UV-irradiated U-2OS cells in any of more than 30 heterokaryons that we examined. To deter-

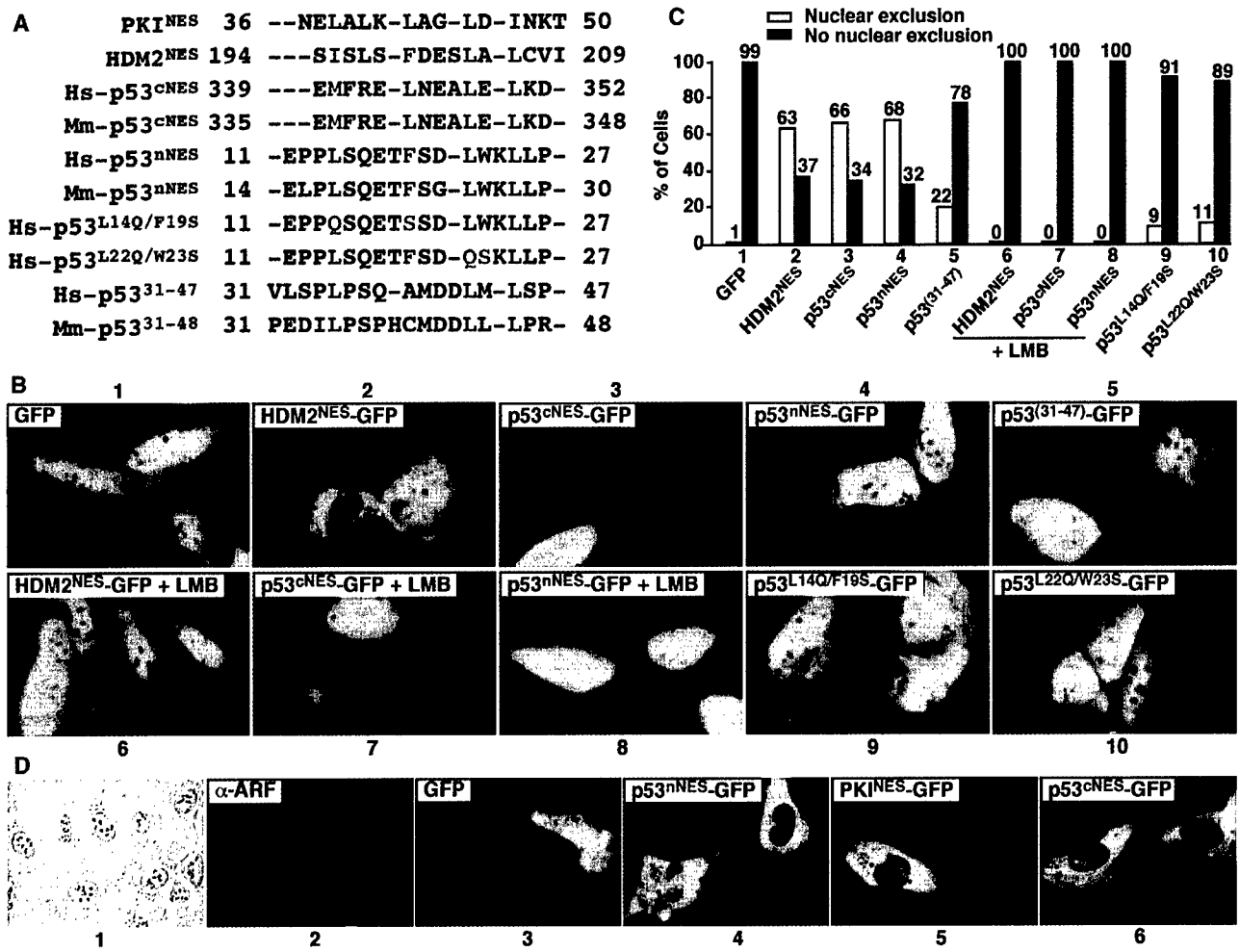

Fig. 3. The NH_2-terminal domain of p53 contains a functional NES. (**A**) Comparison of the NES sequences from human cAMP-dependent protein kinase inhibitor (Hs-PKI), HDM2, and the NH_2-terminally (nNES) and a COOH-terminally (cNES) located NESs from human (Hs) and mouse (Mm) p53 proteins. Highly conserved hydrophobic residues are shown in red and mutations are shown in green. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) Plasmids expressing naked GFP and various GFP fusion proteins as indicated were transfected into U-2OS cells. Thirty-six hours after transfection, the subcellular localization of GFP was examined microscopically in live cells. (**C**) Cells with clear nuclear exclusion of GFP were scored as positive. More than 200 cells were counted for each sample. (**D**) MDM2-independent function of the p53 NH_2-terminal NES. U-2OS cells were first infected with Ad-ARF to block possible MDM2 nuclear export (panels 1 and 2). The cells were then transfected with the indicated plasmids, and the subcellular localization of GFP was examined microscopically in live cells (panels 3 to 6).

mine whether endogenously expressed, Ser^{15}-phosphorylated p53 is similarly blocked from export, U-2OS cells were either left untreated or irradiated with UV and then fused with Saos-2 cells. Almost no p53 was detected in untreated U-2OS cells (Fig. 4B). After UV irradiation, there was a substantial increase in total and Ser^{15}-phosphorylated p53. Ser^{15}-phosphorylated p53 was localized mostly to speckles and exhibited a pattern distinct from that detected by anti–full-length p53, suggesting that phosphorylation of p53 is associated with or promotes a change of p53 subnuclear localization. Where there was active p53 nuclear export, as determined by the use of anti–full-length p53 antibody, no Ser^{15}-p53 signal was detected in the recipient Saos-2 cells of more than 30 heterokaryons examined. UV irradiation similarly resulted in a substantial increase in total and Ser^{15}-phosphorylated p53 in MEFs. A portion of p53 detected by anti–full-length p53 was exported reproducibly at a lower level in MEFs than in U2OS cells, possibly resulting from more efficient p53 phosphorylation (thus blocking export) and a lower efficiency of anti–human p53 in recognizing mouse protein. Consistently, Ser^{15}-phosphorylated p53 was completely blocked from nuclear export in more than 50 heterokaryons examined.

Our results reveal a previously unrecognized NES in the NH_2-terminal region of p53 that is required for the nuclear export of p53. The two separate NESs of p53 can functionally collaborate with each other and synergistically mediate protein nuclear export [Web fig. 3 (*20*)]. Having two separate NESs could provide cells with greater versatility in regulating p53 export. The carboxyl-terminal p53 NES is situated within the tetramerization domain, leading to the postulation that regulated p53 tetramerization occludes this NES, thereby ensuring nuclear retention of the active DNA-binding form of p53 (*14*). The amino terminus of p53 contains several sites whose phosphorylation by various DNA damage–activated p53 kinases leads to p53 stabilization and activation (*22*). The inability of Ser^{15}-phosphorylated p53 to undergo nuclear export suggests a previously unrecognised mechanism—inhibitory phosphorylation of a NES—for stabilizing and activating p53 in response to DNA damage. It remains to be determined whether phosphorylation at Ser^{15} alone is sufficient to inhibit p53 export or whether simultaneous phosphorylation at multiple residues, including Ser^{20}, is required. Phosphorylation at Ser^{15} and Ser^{20} has been suggested to cause p53 stabilization by hindering p53-MDM2 binding (*24*). Because p53 can undergo active nuclear export in the absence

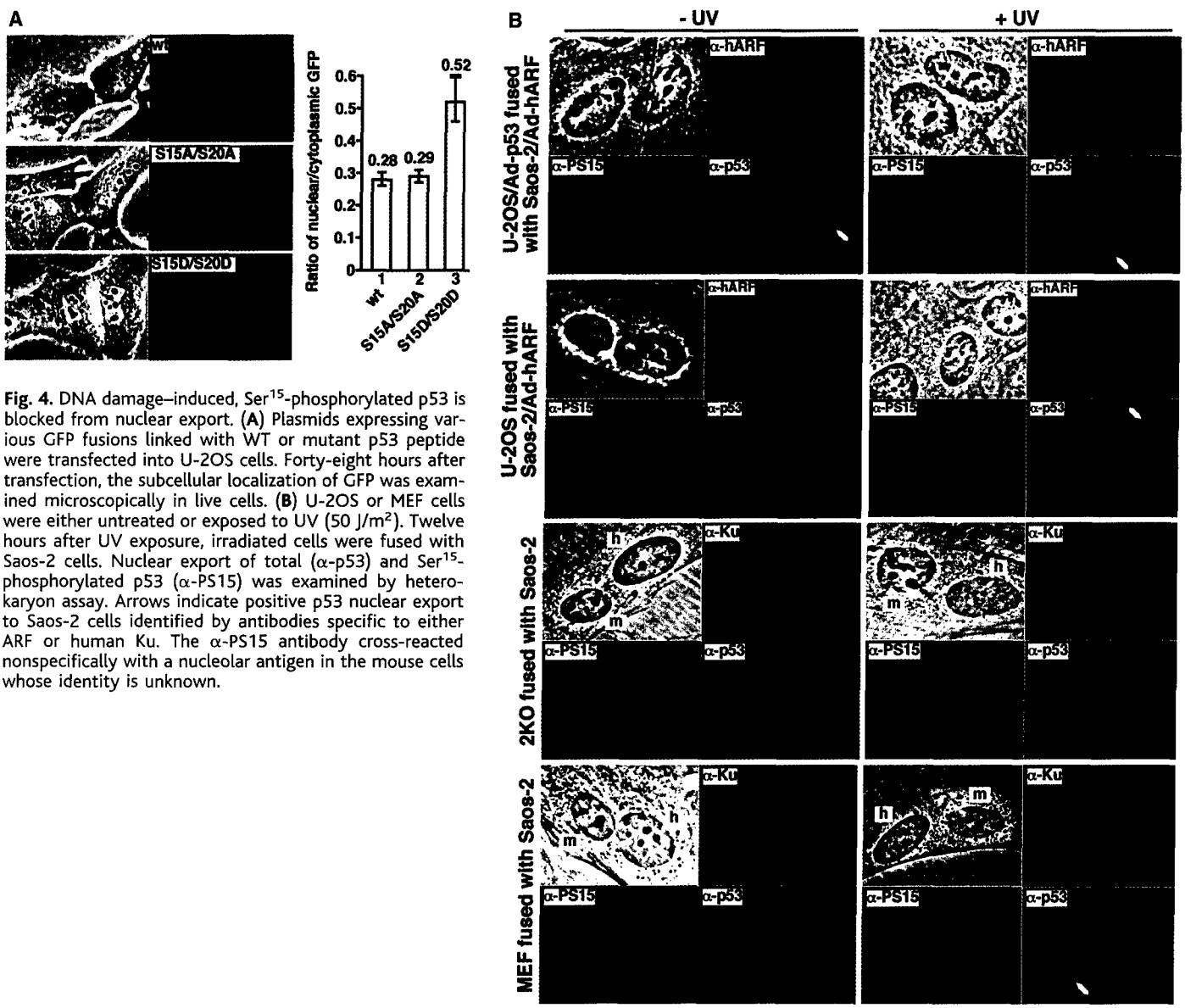

Fig. 4. DNA damage–induced, Ser^{15}-phosphorylated p53 is blocked from nuclear export. (**A**) Plasmids expressing various GFP fusions linked with WT or mutant p53 peptide were transfected into U-2OS cells. Forty-eight hours after transfection, the subcellular localization of GFP was examined microscopically in live cells. (**B**) U-2OS or MEF cells were either untreated or exposed to UV (50 J/m^2). Twelve hours after UV exposure, irradiated cells were fused with Saos-2 cells. Nuclear export of total (α-p53) and Ser^{15}-phosphorylated p53 (α-PS15) was examined by heterokaryon assay. Arrows indicate positive p53 nuclear export to Saos-2 cells identified by antibodies specific to either ARF or human Ku. The α-PS15 antibody cross-reacted nonspecifically with a nucleolar antigen in the mouse cells whose identity is unknown.

of MDM2 (*14*) (Fig. 2), inhibition of MDM2 binding, although preventing p53 degradation, would not block p53 nuclear export and thus would not efficiently accumulate p53 in the nucleus to allow maximal p53 activation. On the other hand, inhibiting p53 nuclear export without breaking its binding with MDM2, although causing the nuclear accumulation of p53, would not reach maximal p53 activation either because MDM2, in addition to its activity in promoting cytoplasmic p53 degradation, can also directly inhibit p53's transactivating activity in the nucleus (*4*). We suggest that DNA damage–induced phosphorylation may achieve optimal p53 activation through the additive and complementary action of both inhibiting MDM2 binding to, and the nuclear export of, p53.

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18. Plasmids expressing WT p53, mutant $p53^{L14Q/F19S}$, $p53^{L22Q/W23S}$, and $p53^{R273H}$ were provided by J. Chen. All other p53 mutants were generated by site-directed mutagenesis with a Quick-Change kit (Stratagene, La Jolla, CA) and verified by DNA sequencing. Cells, cell culture, and procedures for transfection, adenovirus infection, and immunoblotting are described in (*25*). Procedures for indirect immunofluorescence and heterokaryon assay are described in (*11*) except that the incubation time with primary anti-p53 was increased to overnight at 4°C to detect UV-induced p53 in MEFs. Fluorescence images were captured with a cooled charge-coupled device color digital camera (Diagnostic, model 2.2.0) and analyzed on a Macintosh computer with the public domain NIH Image program (version 1.61; available at http://rsb.info.nih.gov/nih-image/). Dilutions and sources of primary antibodies for indirect immunofluorescence are as follows: 0.2 μg/ml for mouse anti-MDM2 (clone SMP14, NeoMarkers, Fremont, CA), 0.04 μg/ml for rabbit anti-MDM2 (N-20, Santa Cruz Biotechnology, Santa Cruz, CA), 0.4 μg/ml (MEFs) or 0.2 μg/ml (other cells) for goat anti-p53 (sc-6243G, Santa Cruz), 1:5000 dilution for affinity-purified rabbit anti–Ser^{15}-phospho-p53 (#9284, New England Biolabs, Beverly, MA), 0.4 μg/ml for mouse anti-ARF (clone 14P02, NeoMarkers), and 2 μg/ml for anti-Ku (p80, clone 111, NeoMarkers). All fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) are diluted to 5 μg/ml. For leptomycin B treatment, cells were treated with 5 ng/ml leptomycin B for 6 hours before cell fusion and/or fixation.
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26. We thank Y. Xu for providing the $p53^{L25Q/W26S}$ embryonic stem cells, S. Jones for the p53-MDM2–deficient MEF cells, C. Finlay and T. Kowalik for the MDM2 and p53 adenoviruses, and J. Chen for p53 and HDM2 plasmids. We also thank J. McCarville and G. White Wolf for technical assistance and C. Jenkins for reading the manuscript. Y.Z. is the recipient of a Career Award in Biomedical Science from the Burroughs Wellcome Fund and a Howard Temin Award from National Cancer Institute. Y.X. is the recipient of a Career Development Award from the U.S. Department of Army Breast Cancer Research Program. Supported by NIH grant CA65572 (Y.X.).

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